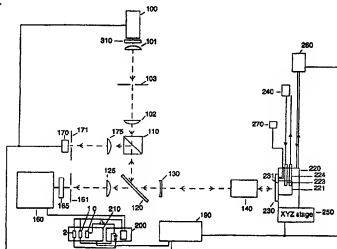




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(54) Title: METHOD AND APPARATUS FOR DETECTION OF FLUORESCENTLY LABELED MATERIALS



## (57) Abstract

Fluorescently marked targets bind to a substrate (230) synthesized with polymer sequences at known locations. The substrate (230) is mounted on an x-y-z translation table (240) during detection. The targets are detected by exposing selected regions of the substrate (230) to light from a light source (100) and detecting the light photons fluoresced therefrom, moving the substrate (230) position, and repeating the exposure and detection steps until the substrate (230) has been completely examined. The device includes a controller (190) for controlling the x-y-z translation table (240) movement for exposure of the selected regions of the substrate (230), the detection of the fluoresced light, and positioning of the substrate (230) to provide proper focusing of the light on the substrate (230) for maximum sensitivity. The resulting data can be used to determine binding affinity of the targets to the specific polymer sequences.

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METHOD AND APPARATUS FOR DETECTION OF  
FLUORESCENTLY LABELED MATERIALS

BACKGROUND OF THE INVENTION

The invention provides a method and associated apparatus for  
5 detecting and analyzing reactions of fluorescently marked materials on a  
single substrate surface.

Certain macromolecules are known to interact and bind to other  
molecules having a very specific three-dimensional spatial and electronic  
distribution. Any large molecule having such specificity can be  
10 considered a target. The various molecules that targets selectively bind  
to are known as probes.

Methods and devices for detecting fluorescently marked targets  
on devices are known. Generally, the devices includes a microscope and a  
monochromatic or polychromatic light source for directing light at a  
15 substrate. A photon counter detects fluorescence from the substrate,  
while an x-y translation stage varies the location of the substrate. A  
computer controls the movement of the x-y translation table and data  
collection. Such devices are discussed in, for example, U.S. Pat No.  
5,143,854 (Pirrung et al.) incorporated herein by reference for all  
20 purposes. See also PCT WO 92/10092 incorporated herein by reference for  
all purposes.

Light from the light source is focused at the substrate  
surface by manually adjusting the microscope. Manual adjustment is, on  
occasion, time consuming and inconvenient. Moreover, due to inherent  
25 imperfections present in the x-y translation table and substrate, there is  
a possibility that the substrate will be out of focus as it is moved from  
one region to another. As a result, the data collected may be  
misrepresented.

Also, temperature sometimes impacts a chemical reaction  
30 between targets and probes. Generally, targets are more active or form  
stronger bonds at lower temperatures; while the converse is true at higher  
temperatures. However, if the temperature is too low, the binding  
affinity of the target may become excessively strong, thus causing target  
to bind with complements (matches) as well as non-complements  
35 (mismatches). Hence, the ability to control temperature may effect  
optimum binding between the targets and probes while minimizing  
mismatches.

In addition, the microscope detection devices are uneconomical  
to use. Typically, these devices incorporate the use of a microscope and  
40 a multichannel scaler, both of which are costly.

From the above, it is apparent that an improved method and apparatus for detecting fluorescently labeled targets on a substrate are desired.

#### SUMMARY OF THE INVENTION

5           Methods and devices for the detection of fluorescently labeled targets on a substrate are disclosed. The detection method and devices utilize a substrate having a large variety of probes at known locations on its surface. The substrate, when placed in a confocal detection device, is exposed to fluorescently labeled targets that bind to one or more of  
10   the probes.

          The confocal detection device includes a monochromatic or polychromatic light source, means for directing an excitation light from the light source at the substrate, means for focusing the light on the substrate, means for controlling temperature of the substrate during a  
15   reaction, means for detecting fluorescence emitted by the targets in response to the excitation light by directing the fluorescence through confocal pinholes, and means for identifying the region where the fluorescence originated. The means for controlling the temperature may include a temperature controlled fluid filled flow cell. The means for  
20   detecting the fluorescent emissions from the substrate, in some embodiments, include a photomultiplier tube. The means for focusing the excitation light to a point on the substrate and determining the region the fluorescence originated from may include an x-y-z translation table. Furthermore, translation of the x-y-z table, temperature control, and data  
25   collection are recorded and managed by an appropriately programmed digital computer.

          In connection with one aspect of the invention, methods for analyzing the data collected by the fluorescent detection methods and devices are disclosed. Data analysis includes the steps of determining  
30   fluorescent intensity as a function of substrate position from the data collected; removing "outliers" (data deviating from a predetermined statistical distribution); and calculating the relative binding affinity of the targets from the remaining data. The resulting data are displayed as an image with each region varying in intensity according to the binding  
35   affinity between targets and probes therein.

          By using confocal optics and focusing and temperature regulating techniques in conjunction with the data analysis methods, it is possible to quickly and accurately determine the relationship between structure and activity of certain molecules. Therefore, the potential for  
40   discovering novel probes with desirable patterns of specificity for biologically important targets is dramatically increased.

A further understanding of the nature and advantages of the inventions herein may be realized by reference to the remaining portions of the specification and the attached drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- 5           FIG. 1a shows a detection system for locating fluorescent markers on the substrate;  
           FIG. 1b shows an alternative embodiment of a detection system for locating fluorescent markers on the substrate;  
           FIG. 1c shows another embodiment of a detection system for  
 10   locating fluorescent markers on the substrate;  
           FIG. 2 shows a flow chart illustrating the operation of the detection system;  
           FIGS. 3a-3b show another flow chart illustrating the focusing step of the detection system;  
 15           FIGS. 4a-4b show another flow chart illustrating the data acquisition step of the detection system;  
           FIG. 4c shows the relationship among the counters in the data acquisition board versus time;  
           FIG. 5 shows another flow chart illustrating the method of  
 20   converting data representing photon counts as a function of position to data representing fluorescence intensity level as a function of position;  
           and  
           FIGS. 6a-6b show another flow chart illustrating the data analysis step,

#### 25           DESCRIPTION OF THE PREFERRED EMBODIMENT

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##### I.       Definitions

The following terms are intended to have the following general meanings as they are used herein:

1.       Complementary: Refers to the topological compatibility or  
 40       matching together of interacting surfaces of a probe

molecule and its target. Thus, the target and its probe can be described as complementary, and, furthermore, the contact surface characteristics are complementary to each other.

- 5           2. Probe: A probe is a molecule that is recognized by a particular target. Examples of probes that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.
- 10
- 15           3. Target: A target is a molecule that has an affinity for a given probe. Targets may be naturally-occurring or manmade molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Targets may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of targets which can be employed by this invention include, but are not restricted to, antibodies, cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells, or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Targets are sometimes referred to in the art as anti-probes. As the term targets is used herein, no difference in meaning is intended. A "Probe Target Pair" is formed when two macromolecules have combined through molecular recognition to form a complex.
- 20
- 25
- 30

## II. Introduction

The present invention provides methods and apparatus for obtaining a highly sensitive and resolved image at a high speed. The invention will have a wide range of uses, particularly, those requiring quantitative study of a microscopic region from within a larger region, such as  $1 \mu\text{m}^2$  over  $100 \text{ mm}^2$ . For example, the invention will find application in the field of histology (for studying histochemical stained and immunological fluorescent stained images), video microscopy, or fluorescence *in situ* hybridization. In one application, the invention herein is used to image an array of probe sequences fabricated on a support.

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The support on which the sequences are formed may be composed from a wide range of material, either biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which a sample is located. The substrate and its surface preferably form a rigid support on which the sample can be formed. The substrate and its surface are also chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO<sub>2</sub>, SiN<sub>x</sub>, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or silica.

According to some embodiments, the surface of the substrate is etched using well known techniques to provide for desired surface features. For example, by way of the formation of trenches, v-grooves, mesa structures, or the like, the synthesis regions may be more closely placed within the focus point of impinging light. The surface may also be provided with reflective "mirror" structures for maximization of emission collected therefrom.

Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. In one embodiment, the surface will be optically transparent and will have surface Si-OH functionalities, such as those found on silica surfaces.

The array of probe sequences may be fabricated on the support according to the pioneering techniques disclosed in U.S. Patent No. 5,143,854, PCT WO 92/10092, or United States Application Serial No. 624120 (Attorney Docket Number 16528X-120), incorporated herein by reference for all purposes. The combination of photolithographic and fabrication techniques may, for example, enable each probe sequence ("feature") to occupy a very small area ("site") on the support. In some embodiments, this feature site may be as small as a few microns or even a single molecule. For example, about 10<sup>3</sup> to 10<sup>6</sup> features may be fabricated in an area of only 12.8 mm<sup>2</sup>. Such probe arrays may be of the type known as Very Large Scale Immobilized Polymer Synthesis (VLSIPS™).

The probe arrays will have a wide range of applications. For example, the probe arrays may be designed specifically to detect genetic diseases, either from acquired or inherited mutations in an individual DNA. These include genetic diseases such as cystic fibrosis, diabetes, and muscular dystrophy, as well as acquired diseases such as cancer (P53 gene relevant to some cancers), as disclosed in United States Patent Application Serial Number 08/143,312, already incorporated by reference.

Genetic mutations may be detected by a method known as sequencing by hybridization. In sequencing by hybridization, a solution containing one or more targets to be sequenced (i.e., samples from patients) contacts the probe array. The targets will bind or hybridize with complementary probe sequences. Generally, the targets are labeled with a fluorescent marker, radioactive isotopes, enzymes, or other types of markers. Accordingly, locations at which targets hybridize with complimentary probes can be identified by locating the markers. Based on the locations where hybridization occur, information regarding the target sequences can be extracted. The existence of a mutation may be determined by comparing the target sequence with the wild type.

The interaction between targets and probes can be characterized in terms of kinetics and thermodynamics. As such, it may be necessary to interrogate the array while in contact with a solution of labeled targets. Consequently, the detection system must be extremely selective, with the capacity to discriminate between surface-bound and solution-born targets. Also, in order to perform a quantitative analysis, the high-density volume of the probe sequences requires the system to have the capacity to distinguish between each feature site.

### III. Fluorescent Detection Device

Fig. 1a schematically illustrates a device used to detect fluorescently labeled targets on a substrate. Substrate 230 comprises a number of presynthesized probes on its surface 231. Such probes may be synthesized according to techniques described in U.S. Pat. No. 5,143,854, PCT WO 92/10092 (attorney docket no. 11509-3900), or other techniques. Substrate 230 is preferably transparent to a wide spectrum of light. In some embodiments, substrate 230 is made of a conventional microscope glass slide or cover slip. It is preferable that the substrate be as thin as possible while still providing adequate physical support. Preferably, the substrate is less than about 1 mm thick, or, even more preferably, less than 0.5 mm thick. Typically, the substrate is a microscope glass slide of about 0.7 mm or 700  $\mu$ m thick. In alternative embodiments, the substrate may be made of quartz or silica.

Substrate 230 is mounted on a flow cell 220. Flow cell 220 is a body having a cavity 221 on a surface thereof. The cavity is between about 50 and 1500  $\mu$ m deep with a preferred depth of 1000  $\mu$ m. In other embodiments, the cavity may be less than 50  $\mu$ m deep. The bottom of the



cavity is preferably light absorbing so as to prevent reflection of impinging light. In addition, the flow cell may be impervious to light.

When mounted to the flow cell, the substrate seals the cavity except for inlet port 223 and outlet port 224. According to a specific embodiment, the substrate is mounted to the flow cell by vacuum pressure generated from a vacuum pump 270. Optionally, one or more gaskets may be placed between the flow cell and substrate, and the intervening space is held at vacuum to ensure mating of the substrate to the gaskets.

Reagents, such as fluorescein labeled targets, are injected into the cavity 221 through the inlet port 223 by using a pump 240 or a syringe. The pump may be, for example, a Masterflex peristaltic pump made by Cole-Parmer Instrument Co. or equivalent. Within the cavity, the reagents bind with one or more complementary probes on surface 231 of the substrate. The reagents are introduced into the cavity via inlet port 223 by the pump and exit through the outlet port 224 for recirculation or disposal.

Flow cell 220 permits the substrate to remain in constant contact with reagents during detection, thereby allowing the substrate to be in equilibrium with targets therein. This arrangement also permits the user to manipulate test conditions without dismounting the substrate. In some embodiments, the flow cell provides means for controlling the temperature within the flow cell. The means for controlling temperature may be a recirculating bath device 260 that flows water through channels formed in the flow cell. In the specific embodiment, device 260 is a refrigerated circulating bath with a RS232 interface, catalog number 13270-615 distributed by VWR or equivalent. However, means such as a circulating air device, a resistance heater, a peltier device (thermoelectric cooler) or others may also be employed. Computer 190 monitors and controls device 260, thereby maintaining the flow cell at a desired temperature. Computer 190 may be selected from a wide variety of computers including, for example, a Gateway 486DX computer or a similar appropriately programmed computer.

Controlling the temperature in the flow cell is advantageous because temperature affects the chemical reaction between targets and probes. For example, the bond between the targets and probes is generally stronger at lower temperatures. However, if the temperature is too low, the binding affinity between targets and probes may become excessively strong so as to produce apparent (but erroneous) matches. Thus, temperature can be controlled to maximize the binding affinity of complementary targets while minimizing mismatches.

Flow cell 220 is mounted on a x-y-z translation table 250. X represents the horizontal direction; y represents the vertical direction; and z represents the direction into and away from the microscope objective such that focusing may be performed. In some embodiments, the x-y-z

translation table may be a Pacific Precision Laboratories Model ST-SLO6R-B5M. Movement of the translation table is controlled by computer 190.

A light source 100 generates a beam of light to excite the fluorescently labeled targets in the flow cell. The light source may be an argon laser that generates a beam, which in some embodiments may be a model 2017 or model 161C manufactured by Spectra-Physics. Other lasers, such as diode lasers, helium-neon lasers, dye lasers, titanium sapphire lasers, Nd:YAG lasers, arc lamp, light emitting diodes, any incandescent light source, or other illuminating device may also be employed. The laser is directed at surface 231 through an optical train comprised of various optical elements which will be described below in detail.

Typically, the light source illuminates the substrate with an excitation wavelength that is within the visible spectrum, but other wavelengths (i.e., near ultraviolet or near infrared spectrum) may be used depending on the application (i.e., type of markers). In some embodiments, the substrate is excited with electromagnetic radiation having a wavelength at or near the absorption maximum of the species of label used. Exciting the label at such a wavelength produces the maximum number of photons emitted. For example, if fluorescein (absorption maximum of 488 nm) is used as a label, an excitation radiation having a wavelength of about 488 nm would induce the strongest emission from the labels.

In instances where a multi-labeling scheme is utilized, a wavelength which approximates the mean of the various candidate labels' absorption maxima may be used. Alternatively, multiple excitations may be performed, each using a wavelength corresponding to the absorption maximum of a specific label. Table I lists examples of various types of fluorophores and their corresponding absorption maxima.

Table I

Candidate Fluorophores	Absorption Maxima
Fluorescein	488 nm
Dichloro-fluorescein	525 nm
Hexachloro-fluorescein	529 nm
Tetramethylrhodamine	550 nm
Rhodamine X	575 nm
Cy3 <sup>TM</sup>	550 nm
Cy5 <sup>TM</sup>	650 nm
Cy7 <sup>TM</sup>	750 nm

Candidate Fluorophores	Absorption Maxima
IRD40	785 nm

When exciting probe arrays fabricated according to photolithographic techniques, they may luminesce when excited by the excitation source, even in the absence of fluorescently labeled targets. Consequently, the signal-to-noise ratio of the detection is decreased. This problem is effectively eliminated by employing an excitation source which illuminates the substrate with an excitation wavelength that is greater than 600 nm. For example, a helium-neon laser, which generates an excitation radiation having a wavelength of about 633 nm, may be used. Through experimentation, it has been determined that exciting the probes with radiation at such wavelength reduces the luminosity of the probes. In some cases, the luminosity factor of the probes decreased by about 100 times. Thus, by exposing the probes with targets labeled with, for example, Cy5<sup>TM</sup> having an absorption maximum which is near that of the excitation wavelength, the signal to noise-to-noise ratio can be significantly increased.

The beam generated by laser 100 is typically nearly collimated and nearly Gaussian. However, a spatial filter may be optionally located in front of laser 100 to improve the Gaussian profile of the beam. The spatial filter may comprise a lens 101, a confocal pinhole 103, and a lens 102. Lens 101 and 102, for example, may be 1/2" diameter 50 mm focal length anti-reflection coated plano convex glass lens or equivalent. Both lenses are configured such that both their back focal planes coincide with confocal pinhole 103. Pinhole 103, for example, may have an aperture of 30  $\mu$ m.

Thereafter, the light passes through a beam splitter 110 to a dichroic mirror 120. The beam splitter may be, for example, a non-polarizing 50% beam splitter cube made by Melles Griot model number 03BSC007 or equivalent; while the dichroic mirror may be a LWP-45°S-488R/520T-1025 made by CVI Laser Corp. or equivalent. The functions of the beam splitter cube will later be described in more detail.

In some embodiments, dichroic mirror 120 passes light having a wavelength greater than about 520 nm, but reflects light having a wavelength of about 488 nm. Consequently, the 488 nm light from the laser is reflected by dichroic mirror 120 toward optical lens 130. Optical lens 130, in the specific embodiment, is 1/2" diameter -50mm focal length anti-reflection coated plano-concave glass lens made by Newport or equivalent. The light then passes through a microscope objective 140 to substrate 230 for magnification of the image sample. Microscope objective 140, in some embodiments, may be a 10x 0.3NA microscope objective, but other

magnifications could also be used. In a preferred embodiment, the distance between lens 130 and microscope objective 140 is about 100 mm.

Microscope objective 140 focuses the light on surface 231, thereby exciting the fluorescein labeled targets. Preferably, the microscope objective produces a spot about 2  $\mu\text{m}$  in diameter in its focal plane. The optical train described in the above embodiments produces a 2  $\mu\text{m}$  diameter focal spot when used with a laser which generates a beam diameter of 1.4 mm, such as the Spectra-Physics model 2017.

In alternative embodiments, the 2  $\mu\text{m}$  spot may be easily obtained when other types of light sources with different beam diameters are used. Since the diameter of the focal spot is inversely proportional to the diameter of the collimated beam produced by lens 102, the desired spot size may be achieved by varying the ratio of the focal lengths of the lenses in the spatial filter. Alternatively, a beam expander may be used to expand or compress the beam from the light source to obtain the desired spot size. For example, if the laser is a model 161C, which generates a beam diameter of 0.7 mm, a 2  $\mu\text{m}$  diameter focal spot may be achieved if the focal-length ratio of the focal lengths of the lenses in the spatial filter is 1:2 instead of 1:1. Thus, by varying the ratio of the focal lengths of the lenses in the spatial filter and/or using a beam expander, the appropriate excitation spot size may be achieved from various beam diameters.

In a preferred embodiment, the laser power delivered to the sample is 50  $\mu\text{W}$ . Depending on the light source used, a variable neutral density filter 310 may be inserted between the laser 100 and the optical train to attenuate the power of the laser to the desired power level.

In response to the excitation light, fluorescein labeled targets in the flow cell fluoresce light having a wavelength greater than about 520 nm. The fluorescence will be collected by the microscope objective 140 and passed to optical lens 130. Optical lens 130 collimates the fluorescence and passes it to dichroic mirror 120. In practice, light collected by microscope objective contains both fluorescence emitted by the fluorescein and 488 nm laser light reflected from the surface 231.

The laser component reflected from the substrate is reflected by dichroic mirror 120 back to beam splitter 110. Beam splitter 110 directs the laser component through a lens 175. The lens, in some embodiments, may be a 1/2" diameter 50 mm focal length anti-reflection coated plano convex glass lens made by Newport, but equivalent thereof may be used. Lens 175 focuses the laser component to a photodiode 170. A confocal pinhole 171 is located at the focal plane of lens 175. Confocal pinhole transmits substantially only the reflected light originating from the focal plane of the microscope to photodiode 170; while reflected light originating from out-of-focus planes is blocked. In some embodiments

confocal pinhole 171 has an aperture of 50  $\mu\text{m}$ . Photodiode 170 generates a voltage corresponding to the intensity of the detected light. Photodiode may be, for example, a 13 DSI007 made by Melles Griot or equivalent, or other light detection devices, such as photomultiplier tube or avalanche photodiode. Output from the detection device is used by computer 190 to focus the laser at a point on surface 231 of substrate 230.

As for the fluorescent component, most of it will pass through the dichroic mirror 120 since its wavelength is greater than about 520 nm. The fluoresced light is then focused by a lens 125 to a photomultiplier tube 160 for detecting the number of photons present therein. Lens 125, in a preferred embodiment, is a 1/2" diameter 50mm focal length anti-reflection coated plano convex glass lens made by Newport, but equivalent lens may be used. A confocal pinhole 161 may be located at the focal plane of lens 125. Confocal pinhole transmits fluorescence originating from the focal plane of the microscope objective and filters out light originating from other planes, such as from the glass or reagent. Accordingly, the signal-to-noise ratio of the fluoresced light is increased. Additionally, a filter 165 is preferably located between photomultiplier tube and confocal pinhole 161. In a specific embodiment, the filter transmits light having a wavelength greater than about 515 nm, such as an Omega Optical 515 EFLP. In an alternative embodiment, the filter may transmit light having a wavelength between about 515 and 545 nm, such as a 530 DF30 made by Omega Optical. Thus, photomultiplier tube 160 detects substantially only fluoresced light.

In the specific embodiment, photomultiplier tube 160 is a Hamamatsu R4457P photomultiplier tube with Hamamatsu C3866 preamplifier/discriminator. The Photomultiplier tube generates approximately a 2 mV pulse for each photon detected. Each of these 2 mV pulses is converted to a TTL pulse by the preamplifier/discriminator. The TTL pulses, each one corresponding to a photon detected by the photomultiplier tube, are then collected by a data acquisition board 210. The data acquisition board may be a National Instruments "Lab-PC+" or equivalent.

Data acquisition board 210, typically, contains an Intel 8254 or equivalent counter/timer chip. This chip contains three counters, counter 0, counter 1 and counter 2. Counter 0 controls the operations of counters 1 and 2 for collecting data. Preferably, counter 0 is programmed to generate a square wave with a period which is equal to twice the data acquisition time per pixel. The output of counter 0 is coupled to an external circuit board 200 which provides logic for inverting the square wave. In a preferred embodiment, the inverted output of counter 0 is connected to the gate input of counter 2; while the non-inverted output is connected to the gate input of counter 1.

In some embodiments, the data acquisition board may not be able to read or store the fast 10 ns pulses generated by preamplifier/discriminator (it is too fast for the 8254 chip). To solve this problem, external circuit board 200 may additionally provide means for slowing down the pulses. For example, the logic in external circuit board 200 may convert these pulses to 50 ns pulses with at least a 50 ns interval between pulses.

The output of the C3866 preamplifier/discriminator, via external circuit board 200, is connected to the clock inputs of counters 1 and 2. When counter 1 or counter 2 is gated on, it counts pulses generated by the preamplifier/discriminator; when it is gated off, it ceases to count and computer 190 reads the accumulated number of counts therein. After the computer reads the count from either counter 1 or 2, the counter is re-initialized on the first clock pulse after its gate input goes high. The initialization pulse is about a 50 ns pulse that is generated by the logic in the external circuit board 200 about 50 ns after each transition of the square wave signal from counter 0. The data stored in counter 1 or 2 represents the photon count as a function of substrate position.

After data are collected from a region of the substrate, substrate 230 is moved so that light can be directed at a different region on the substrate. The process is repeated until all regions on the substrate have been scanned. Generally, regions that contain a complementary probe will tend to exhibit a higher photon count than regions that do not contain a complementary probe.

Although the above embodiments have been described for use in detecting emissions of fluorescein excited by an 488 nm argon laser, it will be apparent to those skill in art that other dyes and excitation sources may be used by simply modifying the elements in the optical train. For example, dichroic mirror 120 may be changed accordingly to pass light having a wavelength comparable to the fluorescence peak of the dye used while reflecting light having a wavelength of the excitation source. In addition, filter 165 is changed to pass substantially only light having a wavelength similar to the fluorescence peak of the dye used. In this manner, the detection device can be easily modified to accommodate other types of excitation light and/or dyes.

Fig. 1b illustrates an alternative embodiment of the fluorescence detection device shown in Fig. 1a. Fig. 1b is similar to the one shown in Fig. 1a, and the common elements have been numbered with the same reference numerals. The main difference between this embodiment and that of Fig. 1a is that a photodiode 180 is provided to detect a component of the light generated by laser 100. Light generated by the laser, as in Fig. 1a, is directed at the beam splitter. However, a component of this light is directed to photodiode 180. Photodiode 180 generates a voltage

directly proportional to the laser power. This voltage signal is used by the computer 190 to monitor and control the laser power.

Fig. 1c illustrates an alternative embodiment of the fluorescence detection device. Fig. 1c is similar to the embodiment shown in Fig. 1a, and the common elements have been numbered with the same reference numerals. However, the embodiment in Fig. 1c provides means for detecting a second fluorescent color. A two-color detection scheme is required when two different types of targets, each labeled with a different dye, are exposed to a substrate synthesized with probes. In some embodiments, fluorescein and rhodamine dyes may be used to label two different types of targets respectively. Typically, each dye will have a fluorescence peak at different wavelengths. For example, the fluorescence peak of fluorescein is about 530 nm while that of a typical rhodamine dye is about 580 nm.

To detect the second fluorescent color, a second dichroic mirror 300 is employed. If rhodamine and fluorescein were used, then dichroic mirror 300 is designed to pass light having a wavelength greater than about 570 nm (rhodamine emissions) and reflect light having a wavelength less than about 560 nm (fluorescein emissions). Light with a wavelength less than 560 nm is reflected to a lens 126 and through a confocal pinhole 151. Lens 126 may be equivalent to lens 125; while confocal pinhole 151 may be similar to confocal pinhole 161. Filter 155 transmits this light to a second photomultiplier tube 150. Filter 155 may be an Omega Optical 530DF30 or equivalent that passes light with a wavelength between about 515-545 nm. This ensures that substantially only fluorescein emissions are detected by the photomultiplier 150.

On the other hand, light having a wavelength greater than 570 nm passes through dichroic mirror 300 to a lens 125. Lens 125 directs this light through pinhole 161 and filter 165 to photomultiplier before entering photomultiplier tube 160. Filter 165 may be a Schott OG570 or equivalent which passes light having a wavelength greater than 570 nm, thereby ensuring that substantially only rhodamine emissions are detected by photomultiplier 160.

Output of the preamplifier/discriminator from the photomultiplier tube 150 is processed by the external circuit board 200 before being connected to counters 1b and 2b on the data acquisition board 205. Data collection by counters 1b and 2b are controlled by counter 0 from data acquisition board 210, which generates a square wave to the gated inputs of the counters 1 and 2 via the external circuit board 200 and is similar to that of counters 1 and 2 on data acquisition board 210.

According to the embodiment in Fig. 1c, two fluorescence colors can be detected by employing a second dichroic mirror, photomultiplier tube and associated lens, confocal pinhole and filter. The embodiment

illustrated in Fig. 1c may be expanded by one skilled in the art to detect more than two fluorescence colors, by employing an additional dichroic mirror, photomultiplier tube and associated lens, confocal pinhole, and filter for each additional fluorescence color to be detected.

- 5 Thus, the various embodiments of the present invention can rapidly acquire images with high resolution and a large field of view. In one dimension, the field of view is dictated by the translation stage and can be arbitrarily large (determined by the distance it translates during one integration period). In the other dimension, the field of view is limited by the objective lens. However, this limitation may be eliminated  
10 employing a translation stage for that dimension.

#### IV. Details on the Operation of a Fluorescent Detection Device

- In the specific embodiment, data are acquired continuously along a line which is broken up into data points or pixels. The pixel size  
15 preferably ranges from 1 to 100  $\mu\text{m}$ . Since pixels are preferably square, the distance between scan lines will generally be between 1 to 100  $\mu\text{m}$ . Each of these pixels will be used to form a digital image. The image resolution (i.e., the degree of discernable detail) depends upon the number of pixels that forms the image. The greater the number of pixels,  
20 the closer the digitized data will approximate the original image. Therefore, it is desirable to have as many pixels as possible. However, the number of pixels is directly related to the length of time required to scan the sample. In practice, it is found that having about 16 to 100 pixels per feature (or about 4-10 pixels along the length of the feature)  
25 is preferable to achieve the desired resolution in a reasonable amount of time.

- The number of photons that is detected in each pixel is contingent upon several factors. The most obvious factor, of course, is the amount of fluorescently labeled targets present in the pixel. Other,  
30 but not so obvious, factors include the intensity of the excitation light, length of time that the targets are excited, and quantum efficiency of the photocathode at the fluorescence emission wavelength.

- For example, exciting a region of about 2  $\mu\text{m}$  with 50  $\mu\text{W}$  of power will yield approximately 1600  $\text{W}/\text{cm}^2$  or  $3.9 \times 10^{21}$  photons/(sec  $\text{cm}^2$ ). At this  
35 intensity, the fluorescence rate is  $Q_k k_f / (k_r + k_f) = 1.1 \times 10^6 / \text{sec}$  (see Table II) with a photodestruction rate of  $Q_k k_p / (k_r + k_f) = 36 / \text{sec}$ .

- In a typical substrate synthesized with polymer sequences at about 5 nm apart, approximately  $4 \times 10^4$  molecules/ $\mu\text{m}^2$  or  $1.25 \times 10^3$  molecules will be present in the excitation volume. If it is estimated that about  
40 1% of these sequences bind with fluorescein labeled targets, then approximately 1250 molecules are excited or  $1.4 \times 10^4$  fluorescence photons are generated per second. However, in a typical detection device, only



about 2% of these photons are collected by the microscope objective; while about 98% never even make it into the optical train of the detection device. Of the 2% collected, about 50% are lost in the optical train. Of the remaining photons, only about 10% are detected by the photomultiplier tube due to quantum efficiency of the photocathode at the fluorescein emission wavelength. Thus, approximately  $1.4 \times 10^6$  photons might be counted per second. From the above, it is apparent that these factors affect the number of photons detected at each pixel.

TABLE II

Fluorescein Optical Parameters (Tsien, R.Y., Waggoner, A. Fluorophores for confocal microscopy: photophysics and photochemistry. In <i>Handbook of Biological Confocal Microscopy</i> ; Pawley, J., Ed.; Plenum Press: New York, 1990; pp. 169-178)	
Absorption cross section, $\sigma$ , $\text{cm}^2/\text{molecule}^{-1}$	$3.06 \times 10^{16}$
Fluorescence rate constant, $k_f$ , $\text{s}^{-1}$	$2.2 \times 10^8$
Absorption rate constant ( $1600 \text{ W/cm}^2$ ) $k_a$ , $\text{s}^{-1}$	$1.2 \times 10^6$
Fluorescence quantum efficiency, $Q_f$	0.9
Photodestruction efficiency, $Q_d$	$3 \times 10^{-5}$

In the present invention, it is preferable to detect as many photons as possible, preferably about 1000 photons per pixel in pixels containing complementary probes and targets because the signal-to-noise ratio is equal to the square root of the number of photon counts. Since the number of photons collected will vary according to the power and length of time the targets are excited by the light, the signal-to-noise ratio may be improved by increasing the laser power or length of time the substrate is exposed to the laser or a combination thereof.

Figs. 2-6 are flow charts describing a specific embodiment.

Fig. 2 is an overall description of the system's operation. Referring to Fig 2, the detection system is initialized at step 200. At step 201, the system prompts the user for test parameters such as:

- a) temperature of the substrate;
- b) number of scans to be performed;
- c) time between scans;
- d) refocus between scans;
- e) pixel size;
- f) scan area; and
- g) scan speed.

The "temperature" parameter controls the temperature at which detection is performed. Temperature may vary depending on the type of

polymers being tested. Preferably, testing is done at a temperature that produces maximum binding affinity and minimizes mismatches.

The "number of scans" parameter corresponds to the number of times the user wishes to scan the substrate; while the "time between scans" parameter controls the amount of time to wait before commencing a subsequent scan. In this manner, the user may perform a series of scans, and if desired, each at a different temperature. Preferably, the "time between scans" is chosen to allow the system to reach chemical equilibrium before commencing a subsequent scan.

In an alternative embodiment, means may be provided to increase the temperature at set increments automatically after each scan. Furthermore, the user may optionally choose to refocus the substrate after each scan.

The "pixel size" parameter dictates the size of each data collection point. Generally, the size is chosen, resulting in at least 16 data collection points or pixels per synthesis region ("feature").

"Scan area" parameter corresponds to the size of the substrate to be tested. "Scan speed" parameter sets the length of time the laser excites each pixel. The slower the speed, the higher the excitation energy per pixel which will result in higher fluorescence count per pixel. Thus, increasing the laser power or decreasing the scan speed or a combination thereof will increase the photon count in each pixel. Typically, the scan rate is set to generate approximately a count of 1000 photons for pixels having fluorescently-marked targets.

At step 202, the system initializes the x-y-z translation table by locating the x-y-z stages at their home position. At step 203, the system focuses the laser on the surface 231 of the substrate. At step 204, the system locates the x-y-z table at its start position. At step 205, the system begins to translate the vertical stage, thereby collecting a series of data points over a vertical line at step 206. When a line of pixels has been scanned at step 207, the x-y-z translation table moves the horizontal stage to collect data from the next line of pixels at step 208. The collected data is written to the file as the substrate is repositioned at the top of the next line. Steps 205 through 208 are repeated until data from all regions have been collected. At step 209, the system determines if there are any more scans to be performed according to the set up parameters. If there are, the system at steps 210 and 211 determines the amount of time to wait before commencing the next scan and to either repeat the process from step 203 (if refocusing of the substrate is desired) or 204. Otherwise, the scan is terminated.

Figs. 3a-3b illustrate the focusing step 203 in greater detail. Auto-focusing is accomplished by the system in three phases. In the first phase, the system focuses the laser roughly on the back surface of the substrate. At step 301, laser light is directed at one corner of the substrate. Laser light reflected by the substrate passes through

microscope objective 140 and optical lens 130 to dichroic mirror 120. The dichroic mirror reflects the light to beam splitter 110, which guides the beam to lens 175. Lens 175 focuses the light through a pinhole 171 to photodiode 170. Pinhole 171 is a confocal pinhole, which transmits light that is reflected from the surface located at the focal plane of the microscope objective and blocks light that is reflected or scattered from other surfaces. At step 302, the computer reads the photodiode voltage, which is proportional to the amount of reflected light transmitted through pinhole 171. The flow cell is then moved about 10 microns closer to the microscope objective at step 315, and the process from step 302 is repeated. The loop commencing at step 302 is repeated until the voltage generated by the photodiode has peaked (i.e., the present voltage value is less than the previous voltage value), at which time the laser is roughly focused on the backside of the substrate. Since 10  $\mu\text{m}$  steps are taken, the light is focused slightly inside the substrate.

At step 304, the system continues with the next focusing phase by moving the flow cell closer to the microscope objective. In a preferred embodiment, the distance over which the flow cell is moved is about equal to half the thickness of the substrate. The default distance is 350 mm (1/2 the thickness of a typical substrate used) or a distance entered by the user representing half the thickness of the substrate used. Again, at step 305, photo-diode 170 generates a voltage corresponding to the intensity of the reflected light. Preferably, the flow cell is then moved about 10 microns closer at step 316, and the process from step 305 is repeated. The loop commencing at step 305 is repeated until the voltage generated by the photodiode has peaked, at which time the laser is roughly focused on a point beyond surface 231.

At step 307, the flow cell is moved farther from the microscope objective in steps of about 1  $\mu\text{m}$ . The computer reads and stores the voltage generated by the photodiode at step 308. At step 309, the encoder indicating the position of the focus stage is read, and the resulting data is stored. The encoder determines the location of the focus stage to within about 1-micron. At step 310, the system determines if the present voltage value is greater than the previous value. If it is, the flow cell is then moved about 1 micron farther at step 317. Due to the presence of noise, the process from step 308 is repeated even after the voltage value has peaked. Specifically, the process continues until the voltage generated by the photodiode 104 is less than the peak voltage minus twice the typical peak-to-peak photodiode voltage noise. At step 311, the data points are fitted into a parabola where  $x$  = encoder position and  $y$  = voltage corresponding to the position. Fitting the data points to a parabola gives a more accurate method of focusing than by merely taking the highest point due to the presence of noise in the data. At step 312, the system determines the focus position on surface 231 which corresponds to the maximum of the parabola.

At step 313, the system determines whether all four corners have been focused. If they have been, then the process proceeds to step 314. Otherwise, the system, at step 318, moves the x-y-z translation stage for directing the light at the next corner and returns to step 301. The focussing process beginning at step 301 is repeated until focus has been found at all four corners of the substrate.

At step 314, the system assumes that the substrate is planar. Thus, the focus position of other pixels are obtained by interpolating the values collected from the focusing process. In this manner, auto-focusing for each pixel of the substrate is achieved.

By using the focusing method disclosed herein, the laser is focused on surface 231 of the substrate, which is significantly less reflective than the backside of the substrate. Generally, it is difficult to focus on a weakly reflective surface in the vicinity of a strongly reflective surface. However, this problem is solved by the present invention, thus achieving a more accurate reading of the fluorescently marked targets.

Fig. 4a-4b illustrate the data acquisition process beginning at step 205 in greater detail. In a specific embodiment, data are collected by repeatedly scanning of the substrate in vertical lines until the sample is completely scanned. However, other techniques, such as repeated scanning of the substrate in horizontal lines, bidirectional scanning (acquiring data in both directions), or others, may be employed.

At step 401, the x-y-z translation table is initialized at the start position. At step 402, the system calculates the constant speed at which the vertical stage is to be moved. The speed of the vertical stage is determined from the scan speed information entered by the user. Typically the scan speed is about 10 to 30 mm/sec or a speed at which a photon count of about 1000 photons will be generated for pixels representing regions having complementary probes. At step 403, the system calculates the constant speed at which the focusing stage is to be moved in order to maintain the substrate surface 231 in focus. This speed is derived from the data obtained during the focusing phase.

At step 404, the system calculates the number of pixels per line by dividing the length of the scan line by the pixel size. At step 405, the system initializes counter 0 on the data acquisition board with a value such that a square wave having a period that is equal to twice the data acquisition time per pixel is generated. The period is calculated by dividing the pixel size information entered by the user at step 209 by the speed of the vertical stage derived from step 402. Counter 0 counts down until it reaches zero, at which time a square wave transition occurs, i.e., output of counter 0 goes from low to high or high to low, and the counter is re-initialized.

In a preferred embodiment, counter 1 on the data acquisition board is configured to store the photon counts from the even pixels; while

counter 2 is configured to store counts from the odd pixels, but other configurations may be employed. At step 406, counter 2 is initialized to zero by the rising edge of the first period of the square wave. Thereafter, counter 2 is enabled and begins to collect data at step 407.

5       The system, at step 408, polls counter 0 and compares the present value of counter 0 with its previous value. If the present value is less than the previous value, then counter 2 continues to accumulate photon counts. On the other hand, if the present value in counter 0 is greater than its previous value, a square wave transition has occurred.  
10      The falling edge of the square wave disables counter 2 from counting, thus completing the scan of the first pixel. Simultaneously, counter 1 is initialized because its gate input is coupled to the inverted output of counter 0. The operation of counter 1 will be described in more detail during the discussion on the second pass of the loop beginning at step  
15      406.

      While counter 2 is disabled, the photon count stored in counter 2 is read at step 409. At step 410, the data is written and stored in memory, for example, in the form of a data structure, an array, a table, or other listing means. In the alternative, the data may also be written  
20      to a data file. At step 411, the system determines if there are more pixels in the line to scan. If there are, process repeats the steps beginning at 406. Otherwise, the system proceeds to step 412.

      On the second pass of the loop beginning at step 405, the inverted falling edge (rising edge) of the square wave initializes and  
25      enables counter 1 to collect data at steps 406 and 407 respectively. At step 408, the inverted rising edge (falling edge) of the square wave disables counter 1, and data therein is read at step 409 and written to the computer at step 410.

      In the specific embodiment, the counters are preferably  
30      configured to collect and store data alternately, i.e., when counter 1 collects data, counter 2 stores data. Fig. 4c illustrates the relationship among the count value in counter 0, the square wave, counter 1 and counter 2 versus time.

      At step 411, the system determines if there are more pixels left  
35      to scan. The loop from step 405 through step 411 is repeated until all pixels in the line have been scanned. After each line has been scanned, the system at step 412 calculates a gray scale for imaging. In a preferred embodiment, the gray scale contains 16 gray levels, but a scale of 64, 256, or other gray levels may be used. In alternative embodiments,  
40      a color scale may be used instead of a gray scale. Preferably, the middle of the scale corresponds to the average count value detected during the scan. Thereafter, the raw data points are stored in a data file at step 413. At step 414, the scaled data representing an image of the scanned substrate regions may be displayed on a screen or video display means in

varying shades of gray or colors. Each shade or color corresponds to the intensity level of fluorescence at the respective regions.

While the image of the previous scanned line is being displayed, the system determines if there are any more lines to scan at step 415. If so, the horizontal stage is translated in preparation for scanning the next line at step 416. The distance over which the horizontal stage is moved is equal to about 1 pixel. Simultaneously, the vertical stage is moved to the top of the next scan line. Thereafter, the system repeats the process starting at step 405 for the next scan line. The loop from step 405 to step 415 is repeated until scanning of the substrate is completed. In this manner, the system simultaneously displays and collects data. Upon completion, the system creates a data file wherein the data represents an array of photon counts as a function of substrate position.

By counting the number of photons generated in a given area in response to the excitation light, it is possible to determine where fluorescently marked molecules are located on the substrate. Consequently, it is possible to determine which of the probes within a matrix of probes is complementary to a fluorescently marked target.

According to preferred embodiments, the intensity and duration of the light applied to the substrate is controlled by the computer according to the set up parameters entered at step 201. By varying the laser power and scan stage rate, the signal-to-noise ratio may be improved by maximizing fluorescence emissions. As a result, the present invention can detect the presence or absence of a target on a probe as well as determine the relative binding affinity of targets to a variety of sequences.

In practice it is found that a target will bind to several peptide sequences in an array but will bind much more strongly to some sequences than others. Strong binding affinity will be evidenced herein by a strong fluorescence signal since many target molecules will bind to that probe. Conversely, a weak binding affinity will be evidenced by a weak fluorescence signal due to the relatively small number of target molecules which bind in a particular probe. As such, it becomes possible to determine relative binding avidity (or affinity in the case of univalent interactions) of a probe herein as indicated by the intensity of a fluorescent signal in a region containing that probe.

Semiquantitative data on affinities may also be obtained by varying set up conditions and concentration of the targets in the reagent. This may be done by comparing the results to those of known probe/target pairs.

While the detection apparatus has been illustrated primarily herein with regard to the detection of marked targets, the invention will find application in other areas. For example, the detection apparatus

disclosed herein could be used in the fields of catalysis, DNA, or protein gel scanning, and the like.

VI. Data Analysis System to Determine Relative Binding Strength of Targets

- 5 Before the data file representing an array of photon counts as a function of position is analyzed to determine the relative binding affinity of targets, the data file containing the raw data is scaled to form an image file.

- 10 Fig. 5 illustrates the process for converting or scaling the raw data file to an image file in greater detail. The conversion procedure is started by prompting the operator for the name of data file of interest. At step 501, the system retrieves the specified data file for analysis.

- At step 502, the user directs the system to scale the data either in relative range or absolute range mode. In the absolute range mode, the data is scaled by using the set of values between the minimum and maximum number of photons. If absolute range mode is chosen, the system proceeds to step 504 which will later be described.

- On the other hand, if relative range mode is chosen, the system proceeds to step 503. Scaling the data in relative range is advantageous. Particularly, relative range mode minimizes the effect of aberrations resulting from dirt or other imperfections on the substrate by ignoring a certain percentage of data points at the intensity extremes. From experience, this range is typically from about 1% of the lowest values and 0.03% of the highest values. At step 503, the user enters the range values or in the alternative, the system may provide for default values. For example, if the user enters .03% and 1% as the relative range values and there are 100,000 pixels in the image, the brightest 30 pixels (0.003x100,000) and dimmest 1000 (0.1x100,000) pixels are clipped (clamped to its next highest or lowest value respectively).

- At step 504, the system determines if the dynamic range (i.e., the ratio of the count in the brightest pixel and the dimmest pixel) of the image is high. If the ratio is high, the system prepares the data for logarithmic scaling at step 505. Otherwise, the system proceeds to step 506 where the data will be scaled linearly. In alternative embodiments, the system may prepare the data for square root scaling instead of logarithmic scaling. In this manner, loss of valid data at the low intensities having low photon counts is avoided, thereby increasing the resolution at these intensities.

- At step 506, the system obtains the minimum and maximum photon counts from the data. The range between these two count values is used to scale the data into digitized units. Preferably, the digitized units have a range from 0 to 255, each one representing a gray level or a color. Alternatively, other ranges such as from 0 to 63 may be used. Generally, the average photon count value is placed in the middle of scale.

Upon completion of the conversion process, an image file representing fluorescence intensity is created and stored in memory at step 507. At step 508, the system may optionally display the image file. The intensity level of the displayed image varies from region to region, according to the binding affinity of the targets to the polymer sequence therein. The brightest signals typically represent the greatest binding affinity; while signals of lesser intensity represent lesser degrees of binding affinity.

As described, data are collected over regions substantially smaller than the area in which a given polymer or feature is synthesized. For example, the length of a pixel is generally 1/4 to 1/10 the length of a feature (or the area of a pixel is 1/16 to 1/100 the area of a feature). Hence, within any given feature, a large number of fluorescence data points or pixels are collected.

A plot of the number of pixels versus the fluorescence intensity for a scan of a substrate synthesized with probes when it has been exposed to, for example, a labeled antibody will typically take the form of a bell curve. However, spurious data are observed, particularly at higher intensities. Since it is preferable to use an average of the fluorescence intensities over a given synthesis region in determining the relative binding affinity, these spurious data points will tend to undesirably skew the data.

Figs. 6a-6b illustrate one embodiment of the system which provides both for the removal of these undesirable spurious data points and the determination of the relative binding efficiency of the sample from an average of the remaining data points.

Referring to Fig. 6, the system is initialized by requesting the user to enter the name of a image file of interest. At step 601, the system retrieves the image file and displays the image on the computer screen and prompts the user to click the mouse on each of the four corners of the synthesis area. Next, at steps 603 and 604, the system prompts the user for the number of cells located horizontally and vertically on the substrate. From the information entered by the user and the information contained in the image file, the system creates a computer representation of a histogram for each cell at step 605. The histogram (at least in the form of a computer file) plots the number of pixels versus intensity.

At step 606, the main data analysis loop is performed for each synthesis site. Analyzing the histogram for the respective synthesis site, the system calculates the total intensity level and number of pixels for the bandwidth centered around varying intensity levels. For example, as shown in the plots to the right of step 606, the system calculates the number of pixels in the bandwidth using a boxcar averaging technique. This process is then repeated until the entire range of intensities have been scanned. At step 607, the system determines which band has the highest total number of pixels. The data from this band is used to derive



statistical data for each synthesis site. The statistical data include the peak value, mean intensity and standard deviation of intensity level. Thus, data that are beyond this band are excluded from the statistical analysis. Assuming the bandwidth is selected to be reasonably small, this procedure will have the effect of eliminating spurious data located at both the higher and lower intensity levels. This loop is repeated until all the cells have been processed.

At step 610, an image in the form of a grid representing the substrate is displayed. Each block in the grid represents a region synthesized with a polymer sequence. The image intensity of each region will vary according to the binding affinity between the polymer sequence and targets therein. Statistical data, such as the peak and average intensity corresponding to each region are also displayed.

To improve imaging, pixels located at transitions between synthesis regions are ignored. The image, in some instances, requires only one pixel space between the cells when the transition of the intensity between the synthesis regions is sharp and distinct. However, if the transition is fuzzy or smeared, the user, at step 611, can select a greater number of pixel spaces between the cells to increase image resolution. If the user enters a value indicating that a greater number of pixel spaces is desired, the system at step 616 reformats the image accordingly.

At step 612, the system retrieves the file created during the synthesis process of the substrate being analyzed. The synthesis file contains sequence information as a function of location. The system integrates the synthesis file with the image file and sorts the data therein. Through this process, the molecular sequence of complementary probes and the intensity as a function of location is available.

Further, the user, at step 614, may analyze a specific synthesis region within the grid. If instructed, the system will display the corresponding substrate position, number of photons, number of pixels, and the molecular sequence at that synthesis site. The data analysis software also provides the user with many functions which are common to image processing, such as magnification and image enhancement.

#### 35 V. Conclusion:

The present invention provides greatly improved methods and apparatus for detection of intensity of fluorescence on a substrate. It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those skilled in the art upon reviewing the above description. Although the detection apparatus has been illustrated primarily herein with regard to the detection of marked targets, it will readily find application in other areas. For example, the detection apparatus disclosed herein could be used in the fields of catalysis, DNA or protein gel scanning, and the

like. The scope of the invention should, therefore, be determined not with the reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

WHAT IS CLAIMED IS:

1. An apparatus for imaging a probe array located on a substrate, said apparatus comprising:
  - a body for immobilizing said substrate, said substrate
  - 5 comprising at least a first surface having said primary array thereon;  - an electromagnetic radiation source for generating excitation radiation having a first wavelength;
  - an optical train for directing said excitation radiation at a first region on said probe array to cause labeled material thereon to emit
  - 10 a response radiation, said response radiation having a second wavelength, said second wavelength different from said first wavelength;
  - a detector for sensing radiation, said optical train directing said response radiation to said detector, said detector generating a signal proportional to the amount of radiation sensed thereon, said signal
  - 15 representing an image associated with said first region;
  - a translator for allowing said excitation radiation to excite a second region;
  - a processor for processing and storing said signal; and
  - 20 a focuser for automatically focusing said substrate in a focal plane of said excitation radiation.
2. The apparatus as recited in claim 1 wherein said body comprises:
  - a mounting surface;
  - a cavity in said mounting surface, said first surface mated to
  - 25 said mounting surface for sealing said cavity, said sample being in fluid communication with said cavity, said cavity having a bottom surface comprising a light absorptive material;
  - an inlet and an outlet being in communication with said cavity such that fluid flowing into said cavity for contacting said sample flows
  - 30 through said inlet and fluid flowing out of said cavity flows through said outlet; and
  - a temperature controller for controlling the temperature in said cavity.
3. The apparatus as recited in claim 3 wherein said
- 35 temperature controller comprises a thermoelectric cooler.
4. The apparatus as recited in claim 1 wherein said excitation source is a laser.
5. The apparatus as recited in claim 1 wherein said first wavelength is selected to approximate the absorption maximum of the said
- 40 labeled material used.

6. The apparatus as recited in claim 1 wherein said first wavelength is about 488 nm.

7. The apparatus as recited in claim 6 wherein said labeled material has a absorption maximum of about 488 nm.

5 8. The apparatus as recited in claim 1 wherein said first wavelength is greater than about 600 nm.

9. The apparatus as recited in claim 8 wherein said excitation source comprises a helium-neon laser.

10 10. The apparatus as recited in claim 8 wherein said labeled material has a absorption maximum of about 650 nm.

11. The apparatus as recited in claim 1 wherein said optical train comprises:

a spatial filter comprising of a first lens and a second lens and a first confocal pinhole located between said first and said second lenses;

15 a beam splitter cube;  
a dichroic mirror for passing radiation of about said second wavelength and reflecting radiation of about said first wavelength;  
an optical lens; and  
20 a microscope objective for directing said light at said substrate.

12. The apparatus as recited in claim 11 wherein said focuser comprises:

a photomultiplier for generating a voltage representing an intensity of said excitation radiation reflected from said substrate; and  
25 a focusing lens for focusing said reflected radiation from said optical train at said photodiode;

means for moving said substrate relative to a microscope objective until said light detected from said substrate substantially  
30 reaches a maximum.

13. The apparatus as recited in claim 12 wherein a second confocal pinhole is located between said focusing lens and said photodiode.

35 14. The apparatus as recited in claim 12 wherein said means for moving is said translator, said translator being an x-y-z translation stage.

15. The apparatus as recited in claim 1 wherein said detector comprises:

- a photomultiplier tube; and
  - a lens for focusing said response radiation collected by said
- 5 optical train at said photomultiplier tube.

16. The apparatus as recited in claim 15 wherein a third confocal pinhole is located between said focusing lens and said photodiode.

17. The apparatus as recited in claim 15 wherein said

10 photomultiplier tube is coupled to a means for collecting pulses generated by said photomultiplier tube in response to said response radiation, said means for collecting pulses communicating with a programmable computer for storing and analyzing said pulses.

18. The apparatus as recited in claim 1 wherein said

15 translator and said focuser comprise a x-y-z translation table, a flow cell mounted on said x-y-z translation table, said flow cell comprising a mounting surface with a cavity therein, said mounting surface comprises means for mounting said substrate thereon and maintaining a sealed relationship with said substrate, said cavity comprises an inlet and an

20 outlet, and said inlet connected to a pump for transferring materials into said cavity and out through said outlet.

19. The apparatus as recited in claim 18 further comprises means for controlling temperature in said flow cell, said means for controlling temperature being a recirculating bath device for circulating

25 water through channels in said flow cell.

20. A method for imaging a probe array located on a substrate, said method comprising the steps of:

- immobilizing said substrate on a body;
- exciting said probe array on said substrate with an excitation

30 radiation having a first wavelength from an electromagnetic radiation source for exciting a first region on said probe array;

- detecting a response radiation having a second wavelength in response to said excitation radiation;
- exciting a subsequent region on said probe array;

35 repeating steps of detecting and exciting a subsequent region until all regions of said probe array has been excited;

- processing and storing said response radiation to generate a 2-dimensional image of said probe array; and
- auto-focusing said substrate is in a focal plane of said

40 excitation radiation.

21. The method as recited in claim 20 wherein said body comprises a mounting surface having a cavity thereon, said substrate immobilized on said mounting surface such that said sample is in fluid communication with said reaction chamber, said reaction chamber comprising  
5 a inlet and a outlet for flowing fluids into and through said reaction chamber.

22. The method as recited in claim 21 wherein said body further comprises a temperature controller for controlling the temperature in said cavity.

10 23. The method as recited in claim 20 wherein said step of detecting comprises the steps of:  
collecting said response radiation through collection optics;  
and  
imaging said response radiation from said collection optics onto  
15 radiation detectors.

24. The method as recited in claim 20 wherein said step of exciting said subsequent region comprises the step of translating said substrate to allow said excitation radiation to excite said subsequent region.

20 25. The method as recited in claim 20 wherein said auto-focusing step comprises the steps of:  
a) focusing a first surface of said substrate;  
b) focusing a second surface of said substrate; and  
c) finely focusing said second surface.

25 26. The method as recited in claim 25 wherein said step of focusing said first surface comprises the steps of:  
directing said excitation radiation at said first surface, said excitation radiation being reflected by said substrate;  
focusing said reflected excitation radiation through a confocal  
30 pinhole;  
detecting said amount of reflected excitation radiation passing through said confocal pinhole, said confocal pinhole configured such that said reflected excitation radiation is located substantially at the center of said confocal pinhole when said first surface is located in  
35 substantially the focal plane of said excitation light;  
determining if said amount of reflected excitation radiation passing through said confocal pinhole has peaked;  
moving said support closer relative to said excitation radiation and repeating the directing, focusing, detecting, determining, and moving

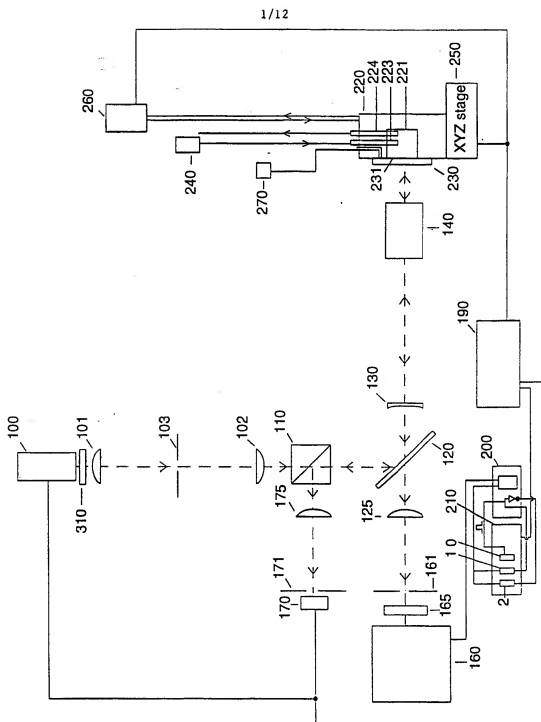
steps until said amount of reflected excitation radiation passing through said confocal pinhole has peaked.

27. The method as recited in claim 26 wherein said step of focusing said second surface comprises the steps of:
- 5 moving said substrate closer relative to said excitation radiation and the distance which the said substrate is moved is equal to about half the thickness of said substrate;
  - directing said excitation radiation at said substrate, said excitation radiation being reflected by said substrate;
  - 10 focusing said reflected excitation radiation through said confocal pinhole;
  - detecting said amount of reflected excitation radiation passing through said confocal pinhole;
  - determining if said amount of reflected excitation radiation
  - 15 passing through said confocal pinhole has peaked;
  - moving said support closer relative to said excitation radiation and repeating the directing, focusing, detecting, determining, and moving steps until said amount of reflected excitation radiation passing through said confocal pinhole has peaked.

28. The method as recited in claim 27 wherein said step of finely focusing said second surface comprises the steps of:
- directing said excitation radiation at said substrate;
  - focusing said reflected excitation radiation through said confocal pinhole;
  - 25 detecting said amount of reflected excitation radiation passing through said confocal pinhole, said confocal pinhole configured such that said reflected excitation radiation is located substantially at the center of said confocal pinhole when said second surface is located substantially in the focal plane of said excitation light;
  - 30 determining if said amount of reflected excitation radiation passing through said confocal pinhole has peaked; and
  - moving said support farther relative to said excitation radiation and repeating the directing, focusing, detecting, determining, and moving steps until said amount of reflected excitation radiation
  - 35 passing through said slit has reached a desired value.

29. The method as recited in claim 25 wherein said steps a-c are repeated for each corner of said probe array.

30. The method as recited in claim 29 further comprising the step of interpolating the focus position of each corner to determine said
- 40 focus position of reach region of said probe array.



**Figure 1a**

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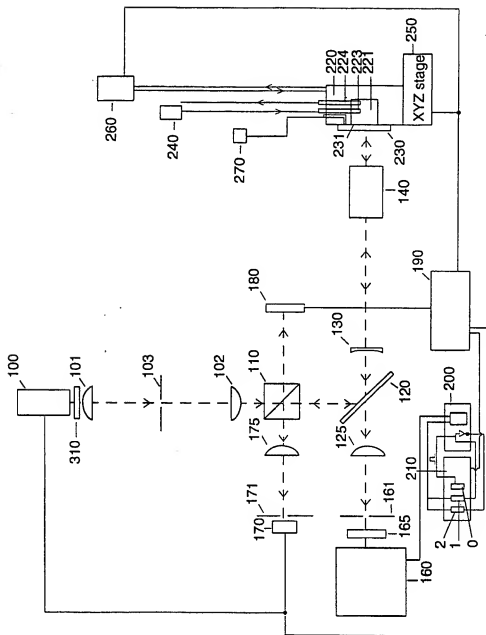


Figure 1b

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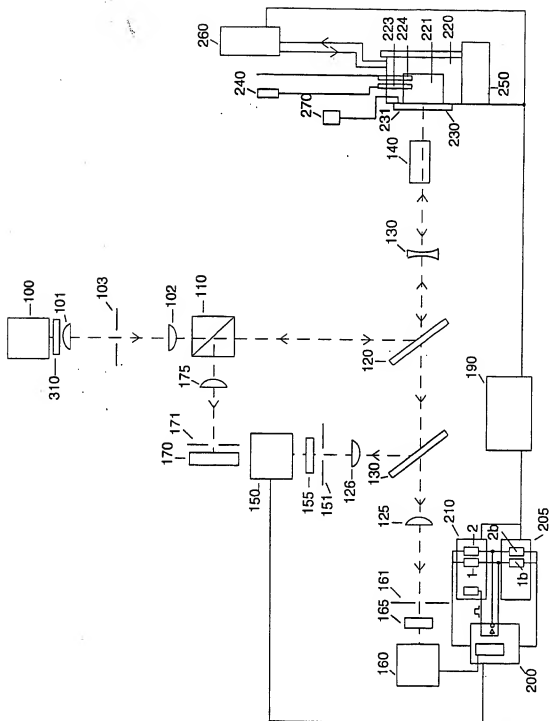


Figure 1c

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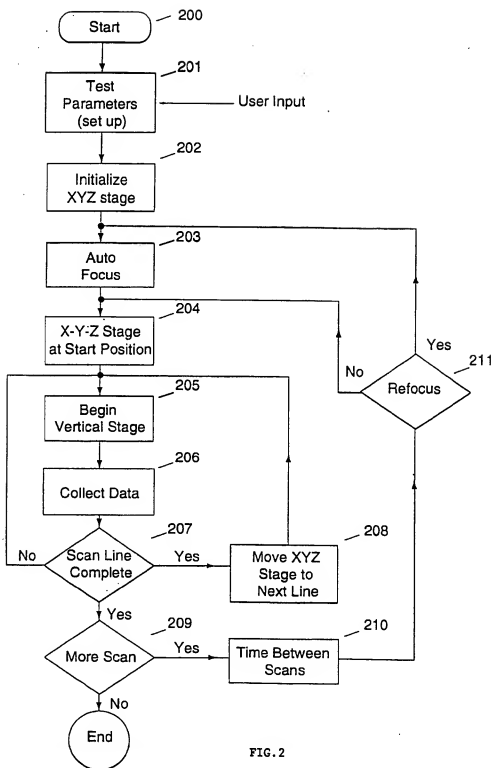


FIG. 2

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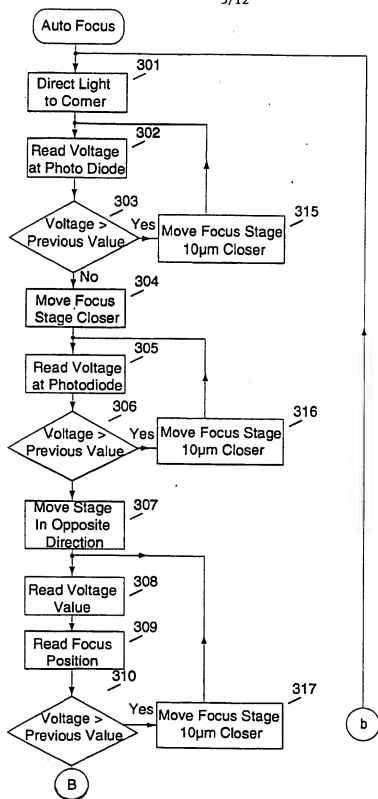


FIG. 3A

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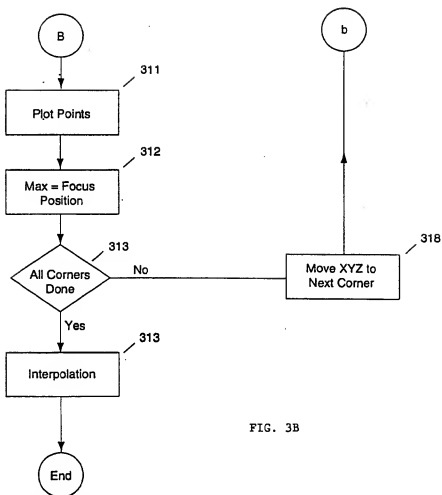
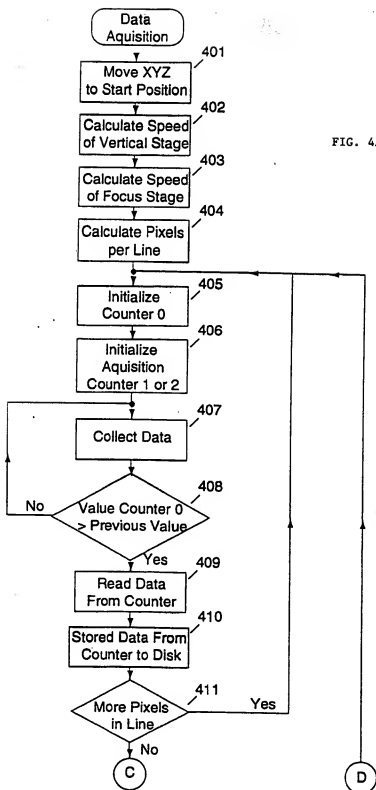


FIG. 3B

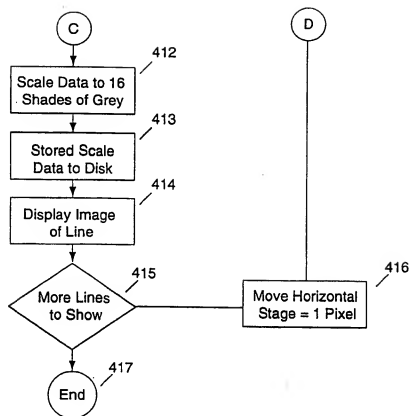
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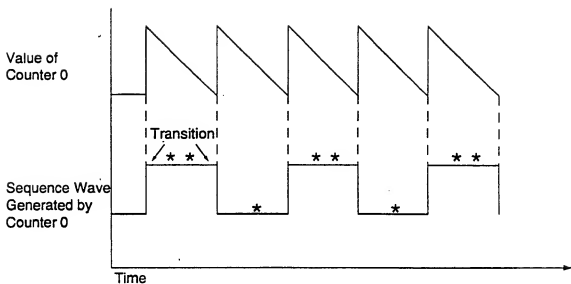
FIG. 4B



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FIG. 4C



\* Counter 1 is enabled to count;  
Data from Counter 2 is read and stored

\* \* Counter 2 is enabled to count;  
Data from Counter 1 is read and stored



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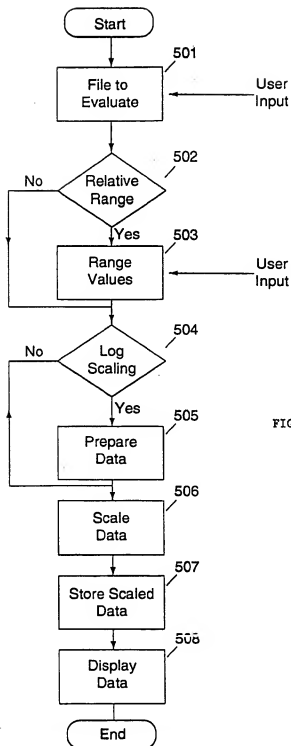


FIG. 5

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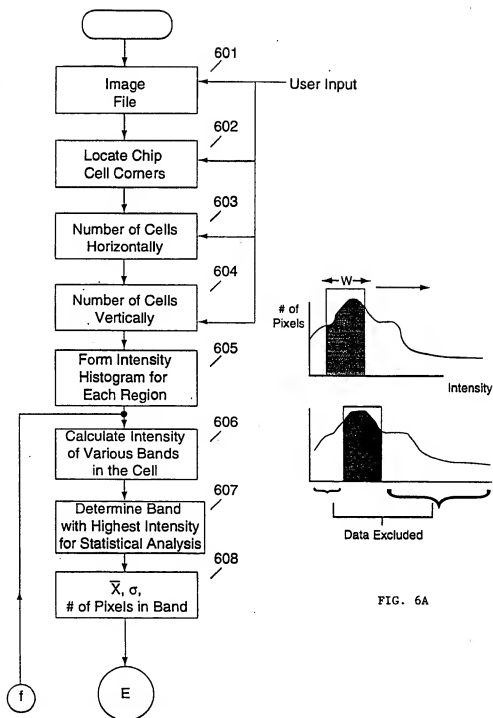
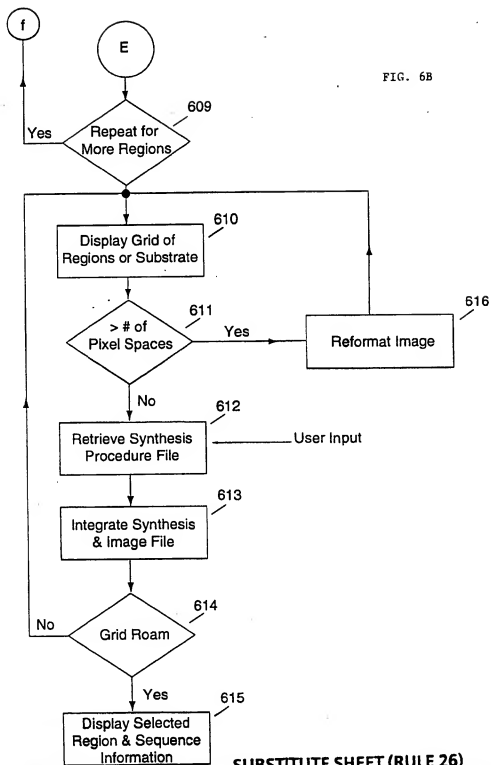


FIG. 6A

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FIG. 6B



SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US95/01886

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/552, 21/64; G01J 1/58

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 250/301.3, 458.1, 459.1, 461.1, 462.1; 356/317; 422/63, 67, 73, 82.08; 436/43, 56, 172, 178, 518, 524, 527

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANALYTICA CHIMICA ACTA, Vol. 227, Issued 01 December 1989, R. Ekins et al, "Development of Microspot Multi-Analyte Ratiometric Immunoassay using Dual Fluorescent-Labelled Antibodies", pages 73 - 96, see entire document.	1 - 30
Y	CLINICAL CHEMISTRY, Vol. 37, No. 11, Issued November 1991, R. P. Ekins et al, "Multianalyte Microspot Immunoassay -- Microanalytical "Compact Disk" of the Future", pages 1955 - 1967, see entire document.	1 - 30

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* "I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "A" document defining the general state of the art which is not considered to be of particular relevance	* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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* "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* "Z" document member of the same patent family
* "O" document referring to an oral disclosure, use, exhibition or other means	
* "P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

15 MAY 1995

Date of mailing of the international search report

22 MAY 1995

 Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/01886

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,342,905 (FUJII ET AL) 03 August 1982, see entire document.	1 - 30
Y	US, A, 4,810,869 (YABE ET AL) 07 March 1989, see entire document.	1 - 30
Y	US, A, 4,844,617 (KELDERMAN ET AL) 04 July 1989, see entire document.	1 - 30
Y	Chemical Abstracts, Vol. 114, No. 26, Issued 01 July 1991, J. Benschop et al, "Confocal compact scanning optical microscope based on compact disk technology", abstract no. 256643p, Appl. Opt. 30(10), 1179-1184, see entire abstract.	1 - 30
Y	US, A, 5,143,854 (PIRRUNG ET AL) 01 September 1992, see entire document.	2, 3, 18, 19, 21, 22
A	SCANNING, Vol. 7, Issued 1985, H. T. M. van der Voort et al, "Design and Use of a Computer Controlled Confocal Microscope for Biological Applications", pages 66 - 78	1 - 30
A	THE JOURNAL OF CELL BIOLOGY, Vol. 105, Issued July 1987, J. G. White et al, "An Evaluation of Confocal Versus Conventional Imaging of Biological Structures by Fluorescent Light Microscopy", pages 41 - 48.	1 - 30
A	JOURNAL OF MODERN OPTICS, Vol. 37, Issued 1990, K. Yamamoto et al, "Features and applications of the laser scanning microscope", pages 1691 - 1701.	1 - 30
A	BIOTECHNIQUES, Vol. 10, Issued 1991, M. A. Quesada et al, "High-Sensitivity DNA Detection with a Laser-Excited Confocal Fluorescent Gel Scanner", pages 616 - 625.	1 - 30

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INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/01886

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

250/201.3, 458.1, 459.1, 461.1, 462.1; 356/317; 422/63, 67, 73, 82.08; 436/43, 56, 172, 178, 518, 524, 527

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN

search terms: optical, fluoresc?, auto focus?, autofocus?, isolat?, confoca?, microscop?, bind?, immobil?, substrat?, surface, support#, carrier#, immunoass?, antigen#, antibod?, scan?